ANTIBODY ENHANCEMENT OF FREE-FLOW ELECTROPHORESIS

H.H.P.Cohly, Ph.D., D.R. Morrison, Ph.D., M.Z. Atassi, Ph.D.

NASA-Johnson Space Center, Houston TX And Department of Biochemistry, Baylor college of medicine, Houston TX.

Summary

The objective of this research is to develop specific T cell clones and antibodies (Abs) to study the efficiency of purifying closely associated T cells using Continuous Flow Electrophoresis System (CFES). Enhanced separation is accomplished by tagging cells first with Abs directed against the antigenic determinants on the cell surface and then with Abs against the Fc portion of the first Ab. This second Ab protrudes sufficiently beyond the cell membrane and glycocalyx to become the major overall cell-surface potential determinant and thus causes a reduction of electrophoretic mobility (EPM). This project has been divided into three phases. Phase one included development of specific T cell clones and separation of these specific clones. Phase two extends these principles to the separation of T cells from spleen cells and immunized lymph node cells. Phase three applies this double antibody technique to the separation of T cytotoxic (Tc) cells from bone marrow.

Background

Analytical Microelectrophoresis

Microelectrophoresis has been used to quantitate the effect of Ab treatment on the EPM of three populations of cells viz. mouse intestinal epithelial cells (IEC), human mononuclear cells (HMNC) and human erythrocytes (7, 8, 12). Comparison of EPM distributions of IEC and HMNC has shown that single Ab treatment caused no change in EPM while double Ab treatment caused considerable reduction. EPM of human erythrocytes has shown that for blood group A1 and N determinants associated with glycocalyx, a single Ab was sufficient to cause reduction while second Ab was needed for cell membrane-associated determinants (Rh blood group) (8, 10). Furthermore, the use of a cationized second Ab resulted in a higher reduction in EPM in a system where the antigen is presumably in the membrane. Single Ab treatment for membrane-associated antigens did not reduce the EPM. It has been proposed that the first Ab is unable to project beyond the glycocalyx of the cell and therefore does not cause a significant decrease in negative charge. However, the addition of second Ab allows the positive charges to extend beyond the glycocalyx resulting in an effective reduction in the normal net negative charge which causes the retardation of cell mobility. This hypothesis is illustrated in Fig. 1. It suggests that a single Ab suffices to affect EPM when the antigen is in the glycocalyx while second Ab is required for effective reduction in EPM when the antigen is in the cell membrane (Fig. 2).

Preparative free-fluid electrophoresis

Studies using heavy water gradient electrophoresis have used the double Ab procedure to separate and purify intraepithelial lymphocytes (IEL) and spleen T
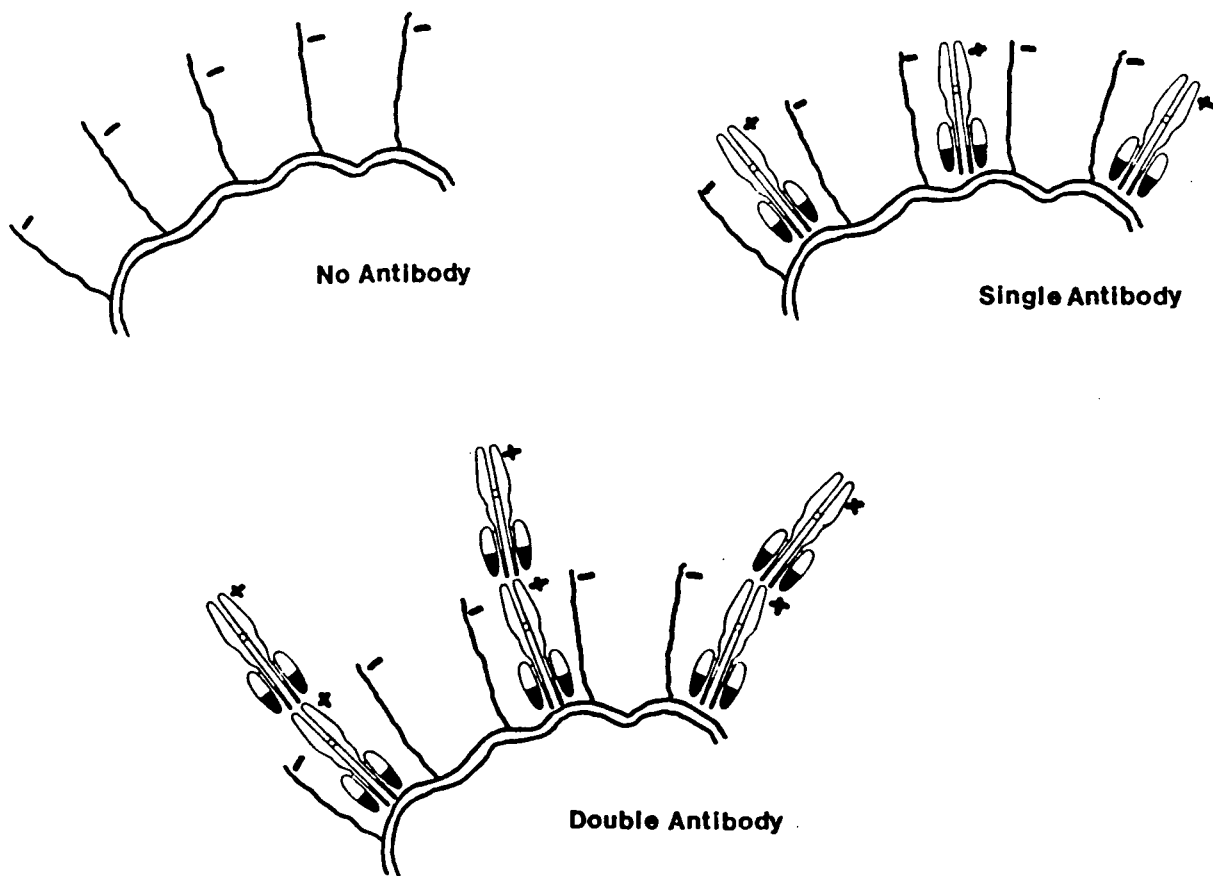
Fig. 1. Schematic representation of the effect of double Ab treatment on the effective charge of cells. A is a cell with its negatively charged glycoprotein strands sticking out. B is a cell which has Ab sticking on to the cell surface where the height of the Ab is similar to the height of the glycoprotein strands. C is a cell where the second Ab is sitting on top of the first Ab and extends beyond the glycocalyx. Only in the last case an effective decrease in EPM occurs.

FIG. 2
LOCATION OF BLOOD GROUP ANTIGENS
ON A
HUMAN RED BLOOD CELL

GLYCOCALYX

A1+ N antigen

CELL MEMBRANE

D antigen
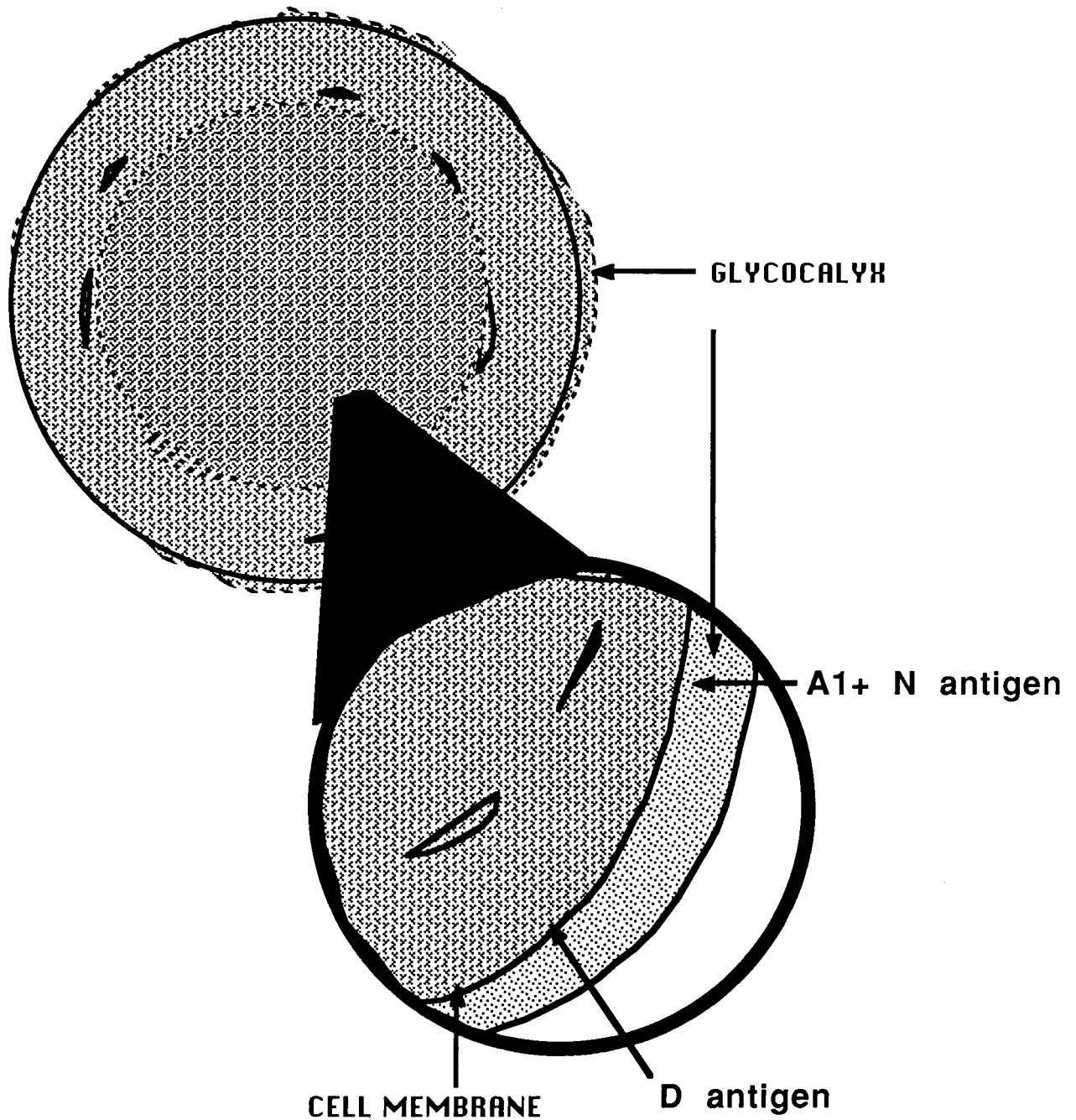
cells (6, 7, 8). IEL from mouse small intestine, which have a 10% epithelial cell contamination after percoll separation, can be further purified by incubating with rabbit IgG (to rat intestinal brush border) followed by incubation with goat IgG to rabbit IgG. This double Ab incubation caused a reduction of EPM of tagged cells sufficient to achieve an electrophoretic fraction virtually devoid of contaminating epithelial cells. In another application T cells were separated from a mixture of Ficoll/Hypaque purified spleen cells by incubating cells with goat anti-mouse total immunoglobulins and then with rabbit anti-goat IgG. With no Ab treatment the fastest moving fraction of T cells had 20% B cell contamination while after double Ab treatment only 2% B cell contamination was found.

One of the main problems encountered in discontinuous heavy water gradient preparative electrophoresis is the small number of cells obtained from the most purified fraction. This restricted analysis of different fractions to phenotyping by cell surface immunofluorescence in lieu of testing their biological activity. This problem may be resolved by using the CFES which offers an increased throughput and resolution for separation of cells without thermoconvection-stabilizing additives.

Approach

Previous methods of cell separation like preparative electrophoresis are limited since the sensitivity of double Ab technique is difficult to quantify by a biological assay. To explore the practical limits of double Ab and CFES a very well characterized model cell system was needed. The rationale was to generate two T cell clones which could differentiate between closely associated antigens. Sperm Whale Myoglobin (SWMb) and Apo-SWMb were selected as the antigens because they only differ in tertiary structure. The EPM of SWMb and Apo-SWMb clones will then be determined. If these two clones do not cross react and have unique EPMs they can be mixed and re-separated on CFES. If there is inadequate difference between the EPM distribution then Ab to known SWMb determinants can be used to increase the separation of SWMb and Apo-SWMb clones. After separation the fractions are tested for their biological activity using proliferative response assay (11). The understanding gained from the model system can then be used to project or predict how useful the double Ab technique will be for other selected target cells.

Phase one included generation of specific T cell clones to different antigenic sites on SWMb or Apo-SWMb. SWMb has five well characterized antigenic sites recognized by antibodies. Site 1 consists of residues 16-21, Site 2 consists of residues 56-62, Site 3 consists of 94-99, Site 4 consists of residues 113-119 and site 5 of residues 146-151 (1, 2). Very little is known about about the molecular parameters involved in the T cell responses to protein antigens. This was mainly due to the inability to prepare specific T cells to well defined protein determinants. T cell cloning has now made it possible to prepare clones to well defined antigenic sites (3, 4, 9). Initial screening for T cell specificity is done with the whole SWMb and the peptide specificity must be determined after second limiting dilution. The specificity of the clones is determined by screening their proliferative response against a pannel of overlapping peptides encompassing the entire SWMb. Six regions of SWMb specifically antigenic to T cells (T sites) were found by a comprehensive synthetic strategy to stimulate SWMb-primed lymph node cells (1, 2, 5, 6, 7). The T sites are localized within the residues 10-22, 46-63, 69-80, 87-100, 107-120 and 137-151 (4, 13).

Experimental Results

Before attempting to generate target clones proliferative assay was performed on Apo-SWMb and SWMb primed lymph node cells which were challenged by synthetic overlapping peptides encompassing the entire SWMb chain. In three separate experiments the response of SJL lymph node cells to Apo-SWMb was always higher than SWMb regardless of whether SWMb or Apo-SWMb was injected. This interesting finding needs to be verified further as it questions current theories about the mechanisms of antigen processing (5).

SWMb clones

After 3 passages T cells specific for SWMb were cloned by serial dilution and 3 clones were obtained viz, A5B8, C2F5 and A2G12. The results of proliferative assay to test the specificity of these clones are shown in Table 2.

Table 2: Proliferative response of SWMb clones to whole SWMB and Apo-SWMb

	Antigen	Optimum dose	Net cpm
A5B8	SWMb	100 ug/ml	15,782
	Apo-SWMb	12 ug/ml	95,734
C2F5	SWMb	6 ug/ml	151,131
	Apo-SWMb	25 ug/ml	187,151
A2G12	SWMb	100 ug/ml	44,629
	Apo-SWMb	25 ug/ml	43,573

Apo-SWMb clones

After first passage T cells specific for Apo-SWMb were cloned by serial dilution. The clones obtained were B4B6, D5A12 and B5C1. These cells are now in the process of being cloned for the second time by serial dilution.

Table 3: Prolferative response of Apo-SWMb clones to SWMb and Apo-SWMb

	Antigen	Optimum Dose	Net cpm
B4B6	SWMb	100 ug/ml	16,650
	Apo-SWMb	25 ug/ml	11,781
D5A12	SWMb	12 ug/ml	4,214
	Apo-SWMb	12 ug/ml	5,928
B5C1	SWMb	6 ug/ml	48,140
	Apo-SWMb	50 ug/ml	48,152

As indicated in Table 2 A5B8 SWMb clone has a higher response to Apo-SWMb than SWMb and in Table 3 the proliferative response of Apo-SWMb clones is similar.

Future Work

The next phase will apply the double Ab technique to enhance the separation of spleen T and B cells. After separation, different fractions will be tested for their phenotypic determinants and mitogenic responses to conconavalin A and lipopolysaccharide The mitogenic response is still a very crude indicator for the purity of T cells or B cells therefore the next step will be to separate T cells from lymph node cells of immunized mice using double Ab and then test their functional activity by a proliferative assay.

Phase three involves the removal of Tc cells from bone marrow then treatment by Ab tagging followed by free-fluid electrophoresis. The procedure would involve the employment of a battery of Abs specific for Tc to determine if single Ab or double Ab treatment is required to remove Tc cells. This approach could have great clinical significance e.g. "purging" of Tc cells from bone marrow for patient therapy and transplantation. Bone marrow transplants would require elimination of only Tc cells (which cause the immune rejection of bone marrow) followed by injection of all remaining cells back into the host.

Conclusions

To date the effect of double Ab treatment to alter the net surface charge of living cells has been documented by analytical electrophoresis. This technique also has been used to purify spleen T and intestinal IEL population by preparative electrophoresis using a heavy water gradient. To better define the extent to which this technique can be applied to different target cells a model T clone system is being developed. Apo-SWMb and SWMb are two almost identical proteins for which specific clones have been generated. These clones will be studied for antigen processing and cell separation on ACE 710 and CFES. Other cell systems proposed to be separated are T cells from spleen, and lymph nodes and Tc from bone marrow. The CFES will be evaluated for efficiency in separating these target cells to determine if standard technique or double Ab enhancement will be sufficient to accomplish the desired cell separation. If not, this data will be used to develop proposals for flight experiments to use CFES in microgravity where the CFES resolution and throughput are enhanced.
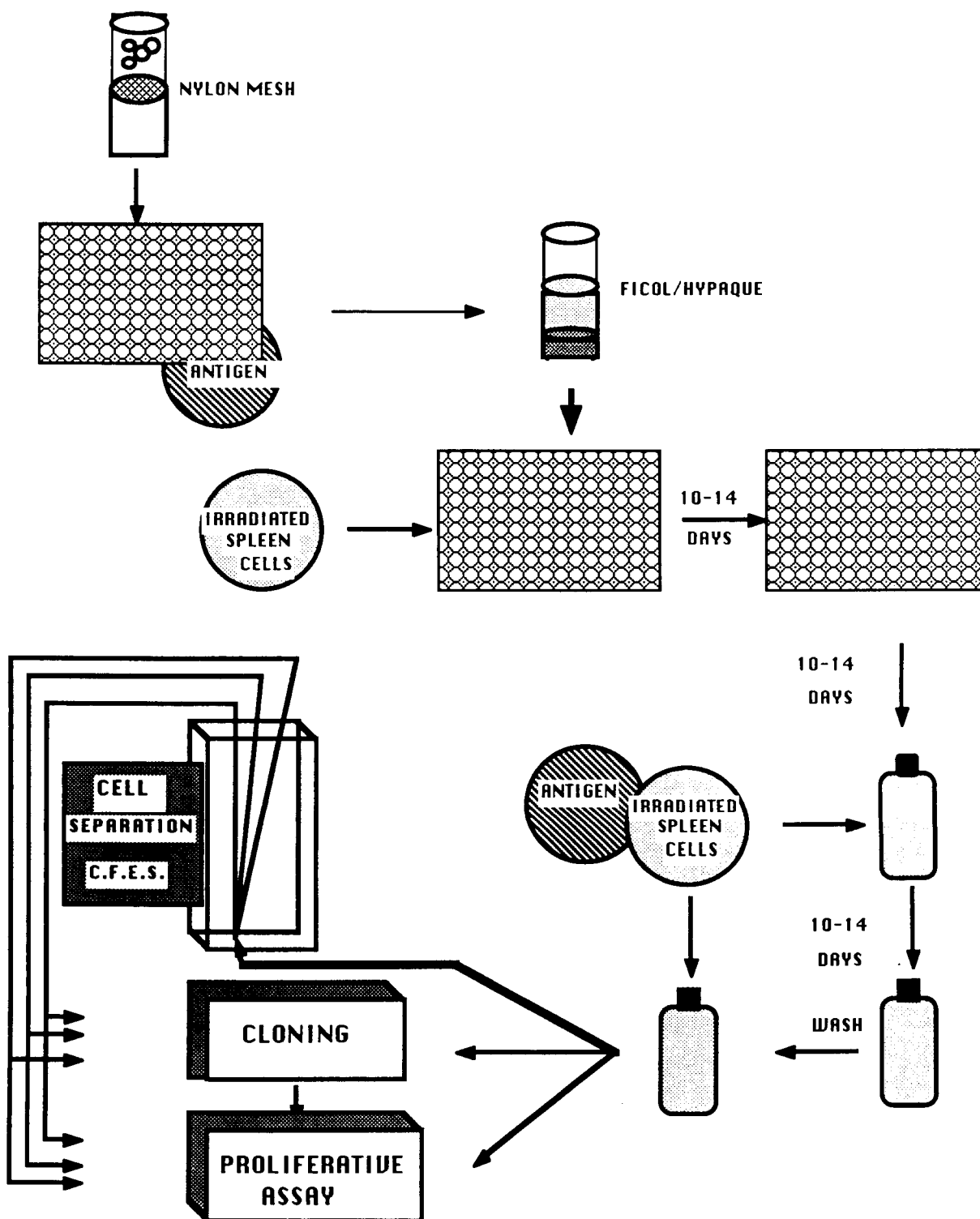
Experimental Procedures

Proliferative Assay

SJL mice were immunized subcutaneously at the base of the tail with 10 ug/mouse of major component (No 10) of Sperm Whale Myoglobin (SWMb) emulsified in Freund's complete adjuvant containing Mycobacterium Tuberculosis, strain H37Ra (Difco, Detroit, MI). Seven days after immunization, the inguinal and periaortic lymph node cells were harvested and the proliferative activity assessed. In a typical assay, lymph node cells suspended in RPMI-1640 containing 1% normal mouse serum were challenged, in triplicate, with various concentrations of antigens and mitogens in 96-well tissue culture plates. After 3 days the cultures were pulsed (18 hrs) with 1 uCi (3H) thymidine and then harvested onto a glass fiber filters for counting by liquid scintilation.

Long term T cell culture

The basic approach to obtain and process long term T cell culture is indicated in Fig. 3. Cultured T cells will be separated on CFES, cloned and tested for

FIG. 3

LONG TERM T CELL CULTURE

NYLON MESH

FICOL/HYPAQUE

ANTIGEN

IRRADIATED SPLEEN CELLS

10-14 DAYS

10-14 DAYS

10-14 DAYS

ANTIGEN IRRADIATED SPLEEN CELLS

WASH

CELL SEPARATION C.F.E.S.

CLONING

PROLIFERATIVE ASSAY

their specificity by proliferative assay. Furthermore, long term T cell culture will be cloned separately and their specificity tested by proliferative assay. Clones specific to SWMb long term T cell cultures were prepared as follows. SWMb primed lymph node cells were suspended in RPMI 1640 supplemented with 10% fetal bovine serum. The cells (2ml/well) were cultured for 4 days in 24-well flat bottom tissue culture treated polystyrene plates with antigen, harvested and then separated on ficoll-hypaque. The cell were recultured with filler cells (X-irradiated, 3300 R, syngeneic spleen cells) in 16 mm wells. Fourteen days later, fluorescein diacetate positive cells were restimulated with the appropriate antigen and filler cells. At 10-12 day intervals, antigen reactive cells were harvested, combined with additional filler cells and restimulated with antigen. T cell clones from protein specific bulk culture were isolated by limiting dilution at a frequency of 1, 0.5 and 0.25 cell/well. The clones were maintained by repeated passage with antigen, filler cells and serum. Specificity of the T cells was determined by their proliferative response. Long term culture of T cells were cocultured with antigens and mitogens in RPMI supplemented with 10% serum. Filler cells were added for macrophage source and the cells were cultured for 3 days and then pulsed with 1 uCi (3H) thymidine as described above.

Preparation and purification of synthetic peptides.

The synthetic peptides were based on the known primary structure of SWMb. The peptides were prepared by solid phase synthesis (11). The primary structure of the SWMb synthetic overlapping peptides was synthesized. Except for the carboxy-terminal peptide 141-153, the peptides are uniform in length (17 residues) and overlap adjacent peptides by 5 amino acids. SWMb sites 1, 2, 3 and 4 which were extended by an additional 5-6 residues (i.e. a total of 13 residues) also were synthesized.

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